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The Actin-Binding Domain of Slac2-a/Melanophilin Is Required for Melanosome Distribution in Melanocytes

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Melanosomes containing melanin pigments are transported from the cell body of melanocytes to the tips of their dendrites by a combination of microtubule- and actin-dependent machinery. Three proteins, Rab27A, myosin Va, and Slac2-a/melanophilin (a linker protein between Rab27A and myosin Va), are known to be essential for proper actin-based melanosome transport in melanocytes. Although Slac2-a directly interacts with Rab27A and myosin Va via its N-terminal region (amino acids 1 to 146) and the middle region (amino acids 241 to 405), respectively, the functional importance of the putative actin-binding domain of the Slac2-a C terminus (amino acids 401 to 590) in melanosome transport has never been elucidated. In this study we showed that formation of a tripartite protein complex between Rab27A, Slac2-a, and myosin Va alone is insufficient for peripheral distribution of melanosomes in melanocytes and that the C-terminal actin-binding domain of Slac2-a is also required for proper melanosome transport. When a Slac2-a deletion mutant (ΔABD) or point mutant (KA) that lacks actin-binding ability was expressed in melanocytes, the Slac2-a mutants induced melanosome accumulation in the perinuclear region, possibly by a dominant negative effect, the same as the Rab27A-binding-defective mutant of Slac2-a or the myosin Va-binding-defective mutant. Our findings indicate that Slac2-a organizes actin-based melanosome transport in cooperation with Rab27A, myosin Va, and actin.

Pigmentation of mammalian hair and skin requires melanosomes, the specialized organelles that produce and store melanin pigments. Melanosomes develop and mature within melanocytes, and mature melanosomes are transported from the cell body of melanocytes to the tips of their dendrites by two distinct motors. Mature melanosomes are first transported to the peripheral region of the cell by long-range, bidirectional, microtubule-dependent movements and are then transferred to actin filaments and carried by unidirectional, short-range, local movement at the cell periphery. Finally, melanosomes are translocated from the dendrites of the melanocyte into adjacent epidermal keratinocytes (24, 29).

Griscelli syndrome (GS) is a rare autosomal recessive disorder characterized by pigment dilution in the skin and hair due to abnormal melanosome transport (i.e., aggregation of melanosomes in the perinuclear region of melanocytes). Most GS patients develop hemophagocytic syndrome, which is characterized by uncontrolled activation of T lymphocytes and macrophages, and some patients exhibit severe neurological impairment (20, 26, 43). Mutations in two different genes on human chromosome 15q21, *MYO5A* and *RAB27A*, have been shown to cause GS (31, 38), and mutations in the corresponding genes (*myo5a*/*dilute* and *rab27a*/*ashen*, respectively) in the mouse cause lighter coat color due to the same defects in actin-based pigment granule transport in melanocytes (33, 48). Myosin Va, the product of *MYO5A*, is an unconventional class V myosin motor protein that moves towards the plus end of actin filaments and functions in intracellular vesicle transport, such as melanosome transport in melanocytes or smooth endoplasmic reticulum transport in Purkinje cells (21, 25, 36, 42, 47, 49). *RAB27A* encodes a small Ras-like GTPase belonging to the Rab family (also called ram) (5, 35), which consists of more than 60 members in humans (4, 39), and Rabs are thought to play essential roles in intracellular vesicle transport by controlling vesicle sorting, tethering, docking, and/or fusion (40, 44, 53). Each Rab protein functions in a specific part of the membrane compartment or at a specific vesicle transport step in a specific cell type. Rab27A plays a critical role in melanosome transport in melanocytes (1, 2, 23, 32, 50), cytotoxic granule exocytosis in T lymphocytes (22, 32, 45), insulin secretion in pancreatic β cells (52), and platelet dense-granule secretion (37, 48).

Recently, a third important player in actin-based melanosome transport, called Slac2-a/melanophilin, has been identified by genetic analysis of coat color mutant mice and biochemical studies. Slac2-a was first identified as a protein homologous to the synaptotagmin-like protein (Slp) family proteins (Slp1 to Slp5) (8, 12, 28), lacking tandem C2 domains at the C terminus (9, 16). It was subsequently identified as a gene product of the *leaden* locus (*mlph*) and is also called melanophilin (30). The Slp and Slac2 families share a highly conserved sequence at the N terminus that has been designated the Slp homology domain (SHD) (16). Biochemical analyses of the SHD have indicated that all SHDs except Slp4-a specifically and directly bind the GTP-bound form of Rab27A but not other Rabs (6, 7, 13, 18, 19, 27, 34, 46, 51, 52). Further biochemical and cellular analyses have indicated that the middle region of Slac2-a directly binds the globular tail and adjacent exon-F of myosin Va and thus that Slac2-a functions as a linker protein between GTP-Rab27A on melanosomes and myosin Va (18, 41, 46, 51). This finding accounts well for the fact that loss of any one of the components of the tripartite protein complex Rab27A–Slac2-a–myosin Va results in the

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same defects in melanosome transport found in *ashen*, *leaden*, and *dilute* mice.

More recently, an additional Slac2 isoform named Slac2-c/MyRIP has been reported and suggested to be involved in retinal melanosome transport through interaction with Rab27A and myosin VIIa (6, 13). Slac2-a and Slac2-c share the same domain structure: an N-terminal SHD, a myosin Va/VIIa-binding domain in the middle region, and a C-terminal conserved domain of unknown function. Although the C-terminal domain of Slac2-a is not involved in recognition of either Rab27A or myosin Va, it has been found to directly bind actin in vitro and to colocalize with actin filaments in cultured cells (13). Functional involvement of the Slac2-a C terminus in melanosome transport, however, has never been investigated. It also remains unknown whether Slac2-a is just a linker protein between Rab27A and myosin Va or has an additional function(s) in melanosome transport.

In this study we identified the critical residue(s) in the Rab27A-binding domain, myosin Va-binding domain (MBD), and actin-binding domain (ABD) of Slac2-a by Ala-based site-directed mutagenesis and created loss-of-function types of Slac2-a that specifically lack Rab27A-, myosin Va-, or actin-binding activity. Introduction of each loss-of-function type of Slac2-a mutant into melanocytes caused abnormal distribution of melanosomes, the same as for GS patients' melanocytes. Our results indicated that formation of a tripartite protein complex by Slac2-a is required but not sufficient for normal melanosome transport and that the C-terminal ABD of Slac2-a is also essential for melanosome transport. Based on our findings, we propose that Slac2-a is a key regulator for melanosome transport and that it functions as a Rab27A effector protein, a cargo recognition mediator for myosin Va, and an anchorage for the actin cytoskeleton.

MATERIALS AND METHODS

Plasmid construction. pEF-T7-Slac2-a or pEGFP-C1-Slac2-a deletion mutants were constructed essentially by conventional PCR as described previously (11, 13, 15, 17). The SHD includes amino acid residues 1 to 153 of mouse Slac2-a, the MBD includes amino acid residues 241 to 405 of mouse Slac2-a, the ABD includes amino acid residues 401 to 590 of mouse Slac2-a; and ΔABD includes amino acid residues 1 to 480 of mouse Slac2-a. Other expression constructs (pEF-FLAG-MC-myosin Va-tail, pEF-FLAG-Rab27A, pEF-HA-Rab27A, and pEGFP-C1-Slac2-a) were prepared as described previously (13, 27).

Mutant Slac2-a plasmids carrying an EA (D378A/E380A/E381A/E382A) or KA (K493A/R495A/R496A/K497A) substitution were produced by two-step PCR techniques with the following mutagenic oligonucleotides with an artificial *Sac*II or *Sph*I site (underlined), respectively, as described previously (14): 5′-CCGCGGCTGTGGCAGCACTGGG-3′ (EA primer 1; antisense), 5′-GCCGCGGCGACACTCAGGAGGA-3′ (EA primer 2; sense), 5′-GGCATGCCTGACGCTGCCGCAGGTGCTCCCGAGGGCTTCACTGTGAG-3′ (KA primer 1; antisense), and 5′-GGCATGCCGATCTTTCTTCC-3′ (KA primer 2; sense). The mutant Slac2-a fragments were subcloned into the pEF-T7 tag vector (11, 15) with appropriate restriction enzyme sites.

Cell culture and transfection. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml at 37°C under 5% CO_2. Lipofectamine Plus (Invitrogen) was used for transfections of plasmids into COS-7 cells as described previously (17). The immortal mouse melanocyte cell line melan-a, derived from a black mouse (3), was kindly provided by Dorothy C. Bennett (St. George's Hospital Medical School, London, United Kingdom) and Katsuhiko Tsukamoto (University of Yamanashi, Yamanashi, Japan). Melan-a cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 2.7 mM HCl, 10% fetal bovine serum, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, 7.5 μg of phenol red per ml, and 0.1 mM 2-mercaptoethanol at 37°C under 10% CO_2. Immediately prior to use, 200 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich) was added to the medium. For subculture, melan-a cells were treated with 250 μg of trypsin per ml–0.1 mM EDTA. Fugene 6 (Roche) was used for transfection of pEGFP-C1 plasmids into melan-a cells according to the manufacturer's instructions.

Immunoprecipitation. T7-tagged Slac2-a, FLAG-tagged MC-myosin Va-tail, and/or hemagglutinin (HA)-tagged Rab27A was coexpressed in COS-7 cells, and recombinant proteins were solubilized at 4°C for 1 h with a buffer containing 1% Triton X-100, 250 mM NaCl, 1mM $MgCl_2$, 50 mM HEPES-KOH (pH 7.2), and appropriate protein inhibitors. T7–Slac2-a was immunoprecipitated with anti-T7 tag antibody-conjugated agarose (Novagen) as described previously (11, 15). Coimmunoprecipitated FLAG–myosin Va-tail, HA-Rab27A, and actin were first detected with horseradish peroxidase-conjugated anti-FLAG tag antibody (Sigma-Aldrich), anti-HA tag antibody (Roche), and antiactin antibody (Santa Cruz Biotechnology), respectively, and the immunoprecipitated T7–Slac2-a proteins were then detected with horseradish peroxidase-conjugated anti-T7 tag antibody (Novagen) as described previously (7, 15, 18). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences). The blots shown in this paper are representative of those from at least two or three independent experiments.

Immunofluorescence and melanosome distribution assay. Melan-a cells (10^5 cells [the day before transfection] per 35-mm-diameter glass-bottom dish; MatTek Corp.) were transfected with 2 μg of a plasmid encoding a green fluorescent protein (GFP)-tagged Slac2-a protein with 3 μl of Fugene 6. At 36 to 48 h after transfection, cells were fixed with 4% paraformaldehyde (catalog no. 168-20955; Wako Pure Chemicals) for 20 min and permeabilized with 0.3% Triton X-100 for 2 min. The cells were then immunostained with anti-Rab27A mouse immunoglobulin G (IgG) (1/50 dilution; BD Transduction Laboratories) and anti-myosin Va rabbit IgG (6.9 μg/ml) (18), followed by anti-rabbit Alexa Fluor 633 IgG and anti-mouse Alexa Fluor 568 IgG (1/5,000 dilution; Molecular Probes) as described previously (10, 27). Images were acquired and pseudocolored with a confocal laser scan microscope (Fluoview; Olympus) and processed with Adobe Photoshop software (version 7.0). Outlines of cells were depicted by Fluoview. Melanosome distribution was assayed by examination of images of transfected melan-a cells (more than 50 cells/dish, three independent dishes for each plasmid) obtained at random. Cells in which more than 50% of melanosomes were present around the nucleus were judged to be aggregated. The absence of melanosomes in the periphery (a phenotype observed in GFP-ABD-transfected cells) was distinguished from perinuclear aggregation of melanosomes by the loss of large clumps of melanosomes around the nucleus.

RESULTS

Identification of critical residues for interactions of Slac2-a with Rab27A, myosin Va, and actin. Our previous studies have demonstrated that Slac2-a (and Slac2-c) contains a Rab27A-binding domain (SHD) at the N terminus, an MBD in the middle region, and an ABD at the C terminus (Fig. 1) (13, 18). Expression of either the Slac2-a SHD or the C-terminal portion (MBD plus ABD [ΔSHD]) in melanocytes results in the formation of GS-like melanocytes (i.e., aggregation of melanosomes in the perinuclear region) (46, 51), possibly by disrupting an endogenous tripartite protein complex between Rab27A, Slac2-a, and myosin Va. Although such experiments clearly show the physiological importance of the SHD and ΔSHD of Slac2-a in melanosome transport, the importance of the SHD-Rab27A and MBD-myosin Va interactions has not been fully demonstrated in vivo, because the possibility that the SHD or ΔSHD fragments may trap molecules other than Rab27A or myosin Va in living cells has not been ruled out. Moreover, since the ΔSHD contains two putative functional domains, the domain(s) that dominantly inhibits melanosome transport (MBD, ABD, or both) has yet to be identified. To resolve these issues, we first attempted to produce a loss-of-function type of Slac2-a by site-directed mutagenesis (Fig. 1A).

Since the closest isoform of Slac2-a, Slac2-c, also possesses the same domain structures (SHD, MBD, and ABD) (Fig. 1B), we performed random Ala-based site-directed mutagenesis,

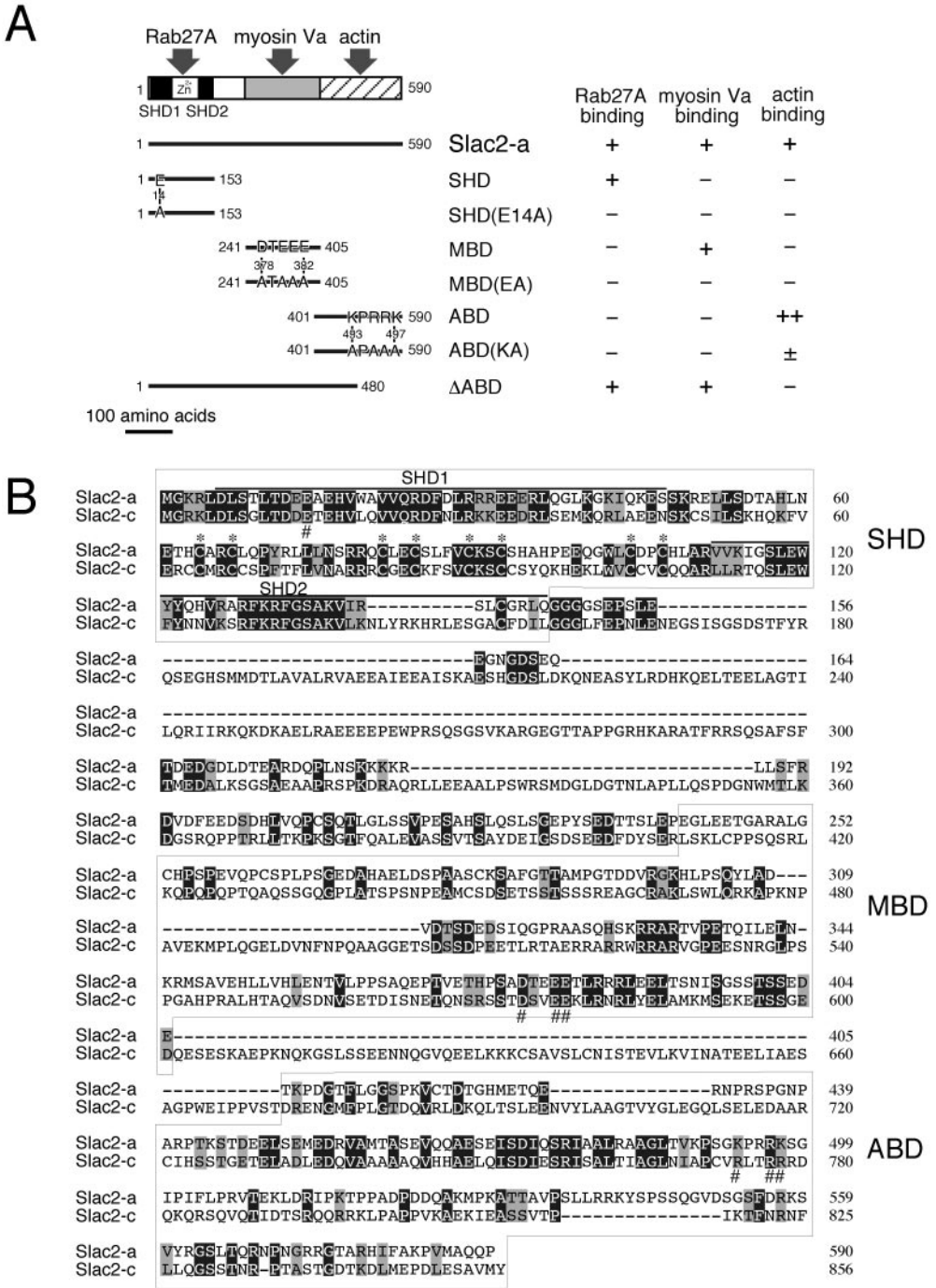

FIG. 1. Domain structures of mouse Slac2-a/melanophilin. (A) Schematic representation of three functional domains of mouse Slac2-a and the deletion or point mutants of Slac2-a used in this study. The SHD is composed of two potential α-helical regions (SHD1 and SHD2; black boxes) separated by two zinc finger motifs (indicated by Zn^{2+}), and it is necessary and sufficient for specific Rab27A/B recognition (7). The MBD and ABD are indicated by the shaded and hatched boxes, respectively (13). The Rab27A-, myosin Va-, and actin-binding activities of each mutant (−, ±, +, or ++) are indicated. (B) Sequence alignment of mouse Slac2-a and Slac2-c. Residues in the sequences that are conserved and similar are shown against black and shaded backgrounds, respectively. The SHD, MBD, and ABD are boxed. The solid lines indicate two SHDs. The pound signs indicate the positions of amino acid residues that are conserved between Slac2-a and Slac2-c, which was mutated in this study (see also panel A). The conserved Cys residues corresponding to two zinc finger motifs are indicated by asterisks. Amino acid numbers are indicated at the right of each line.

especially focusing on the residues that are highly conserved between Slac2-a and Slac2-c. The Rab27A-, myosin Va-, or actin-binding activity of each mutant was evaluated by cotransfection assay in COS-7 cells (11, 15, 18). In brief, T7-tagged Slac2-a mutants and FLAG-tagged Rab27A (or myosin Va tail) were coexpressed in COS-7 cells, and their associations were analyzed by immunoprecipitation with anti-T7 tag antibody-conjugated agarose. Proteins trapped with the beads

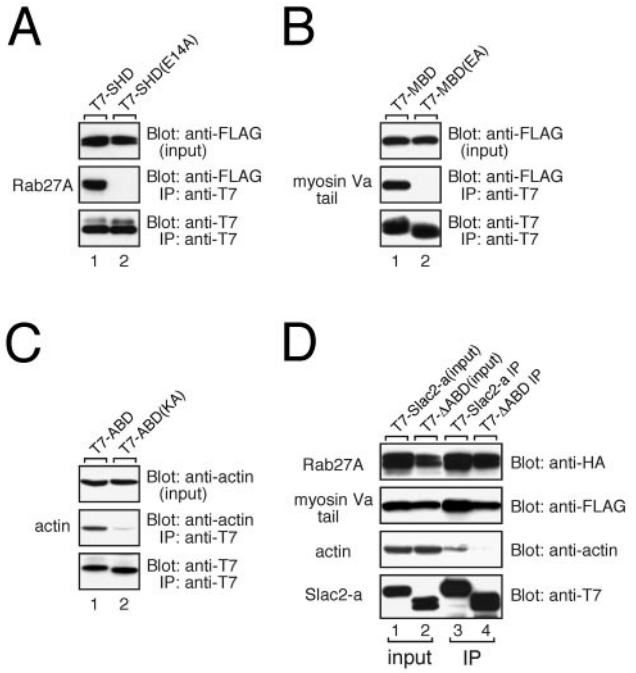

FIG. 2. Identification of critical residues responsible for Rab27A, myosin Va, and actin binding of Slac2-a by Ala-based site-directed mutagenesis. (A to C) Loss of Rab27A-binding activity of the SHD (E14A) mutant (A), loss of myosin Va-binding activity of the MBD (EA) mutant (B), and reduced actin-binding activity of the ABD(KA) mutant (C). T7-tagged Slac2-a mutant and FLAG-tagged proteins (Rab27A or myosin Va tail) were coexpressed in COS-7 cells. Coimmunoprecipitated FLAG-tagged proteins and actin were first detected by anti-FLAG tag antibody and antiactin antibody, respectively (middle panels), and the immunoprecipitated (IP) T7-Slac2-a proteins were then visualized with anti-T7 tag antibody (lower panels). The upper panels show total expressed proteins (1/80 volume of the reaction mixtures; input) used for immunoprecipitation. (D) Deletion of the C-terminal actin-binding site (ΔABD) specifically impaired actin-binding activity but had no effect on Rab27A- or myosin Va-binding activity. pEF-T7-Slac2-a-ΔABD, pEF-HA-Rab27A, and pEF-FLAG-MC-myosin Va-tail were cotransfected into COS-7 cells. Coimmunoprecipitated HA-Rab27A, FLAG-myosin Va, and actin were detected by anti-HA tag, anti-FLAG tag, and antiactin antibodies, respectively (lanes 3 and 4 in the upper three panels). Lanes 1 and 2 represent the total proteins expressed (1/80 volume of the reaction mixtures; input) in lanes 3 and 4, respectively, used for immunoprecipitation. Note that the ΔABD mutant specifically impairs actin-binding activity (lane 4 in the third panel).

were detected with anti-FLAG tag or antiactin (middle panels in Fig. 2A to C) and anti-T7 tag antibodies (bottom panels in Fig. 2A to C). For example, when the Glu-14 of Slac2-a in SHD1 was replaced by Ala (E14A), the mutant SHD(E14A) completely abolished Rab27A-binding activity (Fig. 2A, compare lanes 1 and 2). Since every SHD contains Glu at the corresponding position, the Glu-14 in SHD1 (i.e., first α-helical region) must be critical for recognition of Rab27A (7). Although the MBD and ABD were less conserved between Slac2-a and Slac2-c than the SHD, we identified a conserved acidic cluster (Asp-378, Glu-380, Glu-381, and Glu-382) in the MBD and a basic cluster (Lys-493, Arg-495, Arg-496, and Lys-497) in the ABD as critical determinants for myosin Va binding and actin binding, respectively. The MBD(EA) mutant, carrying the D378A/E380A/E381A/E382A substitution, completely lacked myosin Va-binding activity, and the ABD (KA) mutant, carrying the K493A/R495A/R496A/K497A substitution, exhibited greatly reduced actin-binding activity (Fig. 2B and C, compare lanes 1 and 2). We also prepared full-length Slac2-a mutants carrying a single mutation (E14A, EA, or KA) and confirmed their binding capacity by coexpression assay (T7–Slac2-a, FLAG-myosin Va, and HA-Rab27A) in COS-7 cells (see also Fig. 2D) (13). For example, the full-length Slac2-a(E14A) mutant specifically lacked Rab27A-binding activity, but it bound myosin Va and actin normally, because the MBD and ABD were intact (data not shown), whereas under the same conditions the wild-type Slac2-a coimmunoprecipitated Rab27A, myosin Va, and actin (Fig. 2D, lane 3).

Dominant negative effect of full-length Slac2-a(E14A) and Slac2-a(EA) mutants on melanosome transport. To evaluate the physiological significance of E-14 in the SHD (responsible for Rab27A binding in vitro) and the acidic cluster in the MBD (responsible for myosin Va binding in vitro), we utilized an immortalized black mouse-derived cell line, melan-a, which enables direct observation of the melanosome distribution pattern under a light microscope. In order to identify the functional domains of Slac2-a that are essential for melanosome transport, we established an in vivo assay by transfecting a vector encoding GFP-tagged Slac2-a mutant protein into melan-a cells and observing the melanosome distribution in the transfected cells. From the relative signals of exogenous GFP–Slac2-a and endogenous Slac2-a by immunoblotting (GFP–Slac2-a/endogenous Slac2-a, 1:2) and the transfection efficiency (approximately 10%), the expression levels of transiently expressed Slac2-a proteins were estimated to be about five times as high as those of endogenous Slac2-a, which would be sufficient to overcome the function of endogenous Slac2-a (data not shown).

When GFP alone was transfected into melan-a cells, GFP fluorescence was detected throughout the cells and melanosomes were normally distributed at the periphery of the cell, with both endogenous Rab27A and myosin Va being present on the melanosomes (Fig. 3A to D and yellow in inset of Fig. 3C). Most of the melan-a cells expressing wild-type GFP-tagged Slac2-a showed normal melanosome distribution, and ectopically expressed GFP–Slac2-a was well colocalized with endogenous Rab27A and myosin Va (Fig. 3E to H and white in inset of Fig. 3G), with only a small proportion of the cells (17.8% ± 3.6%) displaying melanosome accumulation in the perinucleus.

When either GFP-SHD or GFP-MBD (i.e., a fragment of the single functional domain of Slac2-a) was expressed in melan-a cells, both SHD (Fig. 4A to D) and MBD (Fig. 5A to D) induced very high rates of melanosome aggregation in the perinucleus (86.5% ± 2.5% and 88.3% ± 4.6% abnormality, respectively), consistent with previous studies (46, 51). Interestingly, endogenous Rab27A protein was often segregated from endogenous myosin Va in SHD-expressing melan-a cells, probably due to occupation of the Slac2-a binding site of Rab27A by ectopically expressed SHD (Fig. 4C, inset). Alternatively, excessive amounts of SHD may also attract Rab27A to unrelated compartments of the cells. The same may be true of MBD expression, and both segregation of myosin Va from endogenous Rab27A and sequestration of endogenous myosin

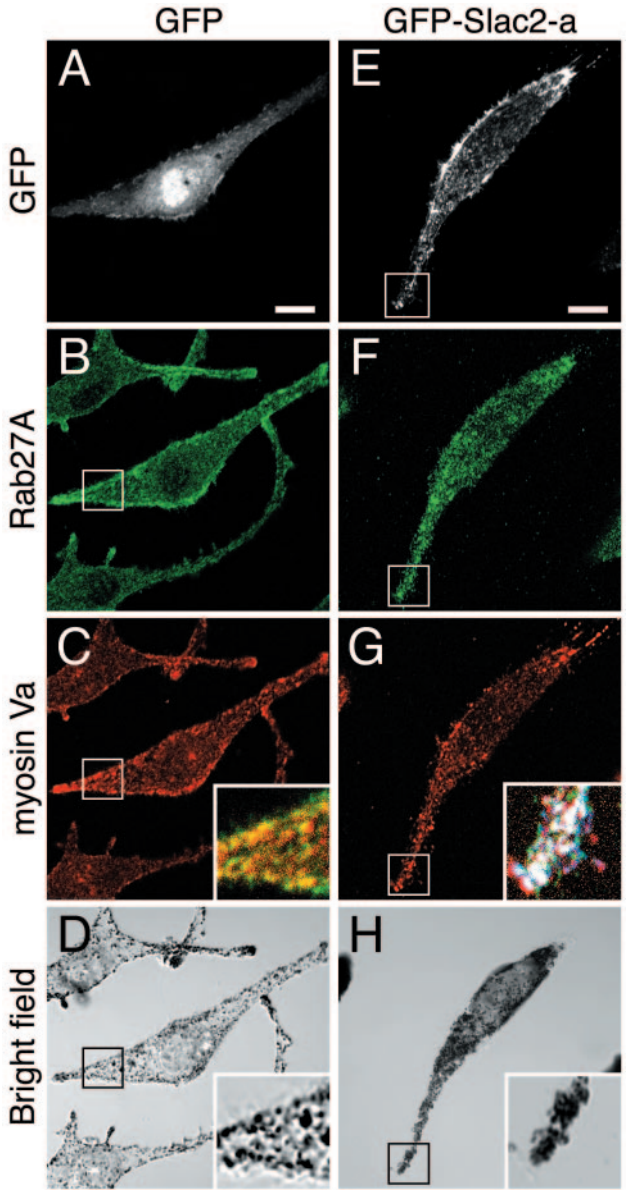

FIG. 3. A black mouse-derived melanocyte cell line, melan-a, exhibits normal melanosome distribution at the cell periphery irrespective of expression of GFP or GFP–Slac2-a. An immortalized black melanocyte cell line, melan-a, was transfected with vectors encoding GFP alone (A to D) or GFP-tagged full-length Slac2-a (E to H). After cells were fixed and permeabilized, they were stained with anti-Rab27A and anti-myosin Va antibodies and visualized with Alexa Fluor secondary antibody conjugates. GFP was localized throughout the cell (A), whereas GFP–Slac2-a was localized at the periphery of the cell (E). Expression of both proteins affected neither the normal distribution of melanosomes at the periphery of the cells, as revealed by bright-field images (D and H), nor localization of Rab27A (B and F) and myosin Va (C and G) on the melanosomes. The inset in panel C is a merged image of Rab27A and myosin Va, where both proteins were colocalized (yellow signals) on melanosomes (see melanosome distribution in the inset in panel D). The inset in panel G is a merged image of GFP–Slac2-a (pseudocolored in light blue), Rab27A (green), and myosin Va (red), where three proteins were colocalized (white signals) on melanosomes (see melanosome distribution in the inset in panel H). Bars, 10 μm.

Va to an unusual part of the cell were also evident in the MBD-expressing cells (Fig. 5C, inset). By contrast, expression of SHD(E14A) or MBD(EA) did not cause either accumulation of melanosomes (9.5% ± 3.8% and 14.9% ± 5.7% abnormality, respectively) or segregation of Rab27A from myosin Va on melanosomes in melan-a cells (Fig. 4E to H and 5E to H). These results indicated that expression of SHD and MBD impaired melanosome transport by specifically disrupting endogenous interaction of Rab27A-SHD and myosin Va-MBD, respectively, in vivo.

Although the single functional domain of Slac2-a with a loss-of-function mutation had no effect on melanosome transport, as described above, full-length GFP–Slac2-a(E14A) lacking Rab27A-binding activity strongly induced aggregation of melanosomes in the perinucleus (90.1% ± 1.0% abnormality), and segregation of endogenous Rab27A from myosin Va seemed to occur (Fig. 4I to L and inset in Fig. 4K). Surprisingly, however, the most striking feature of the cells expressing full-length Slac2-a(E14A) was the reduced immunoreactivity of endogenous myosin Va (compare Fig. 4G and K). It is interesting that a similar phenomenon was observed in *ashen* (mutation in the *rab27a* gene) and *leaden* (mutation in the *slac2-a*/*mlph* gene) mouse-derived melanocytes, in which the immunoreactivity of myosin Va was attenuated (41). This observation may be explained by the notion that an incomplete protein complex containing myosin Va and Slac2-a(E14A), but not Rab27A, which cannot support melanosome transport, may be released from the melanosomes into the cytoplasm and/or be rapidly degraded by unknown mechanisms in melanocytes. Similar results were obtained for the expression of the full-length GFP–Slac2-a(EA) mutant in melan-a cells (Fig. 5I to L): strong aggregation of melanosomes in the perinuclear region, segregation of endogenous Rab27A from myosin Va, and decreased immunoreactivity of endogenous myosin Va.

The ABD of Slac2-a is necessary for actin-based melanosome transport at the cell periphery. Finally, we attempted to determine the function of an uncharacterized domain of Slac2-a, the ABD, in melanosome transport. We previously found that the isolated ABD of Slac2-a colocalized well with actin filaments in B16 melanoma cells and inhibited neurite outgrowth in PC12 cells, possibly by inhibition of actin filament remodeling (13). When GFP-ABD was expressed in melan-a cells, only one-fifth of the melanocytes expressing it showed aggregation of melanosomes (21.7% ± 3.8%) (Fig. 6A to D), and this rate was much lower than with the SHD and MBD (more than 86%). Unexpectedly, however, more than half of the transfected cells (54.7% ± 3.3%) exhibited an unusual phenotype characterized by the exclusion of melanosomes from the cell periphery without prominent clumps of melanosomes around the nucleus (Fig. 6D) even though Rab27A and myosin Va were still present on the melanosomes (Fig. 6C, inset). This phenotype may be attributable to induction of excessive formation of actin bundles in the cell periphery by the ABD of Slac2-a (Fig. 6A). The exclusion of melanosomes from the cell periphery without perinuclear accumulation led us to hypothesize that the ABD is essential for actin-based melanosome transport at the cell periphery not being required for formation of a protein complex with Rab27A and myosin Va (13).

To verify our hypothesis, we produced a deletion mutant

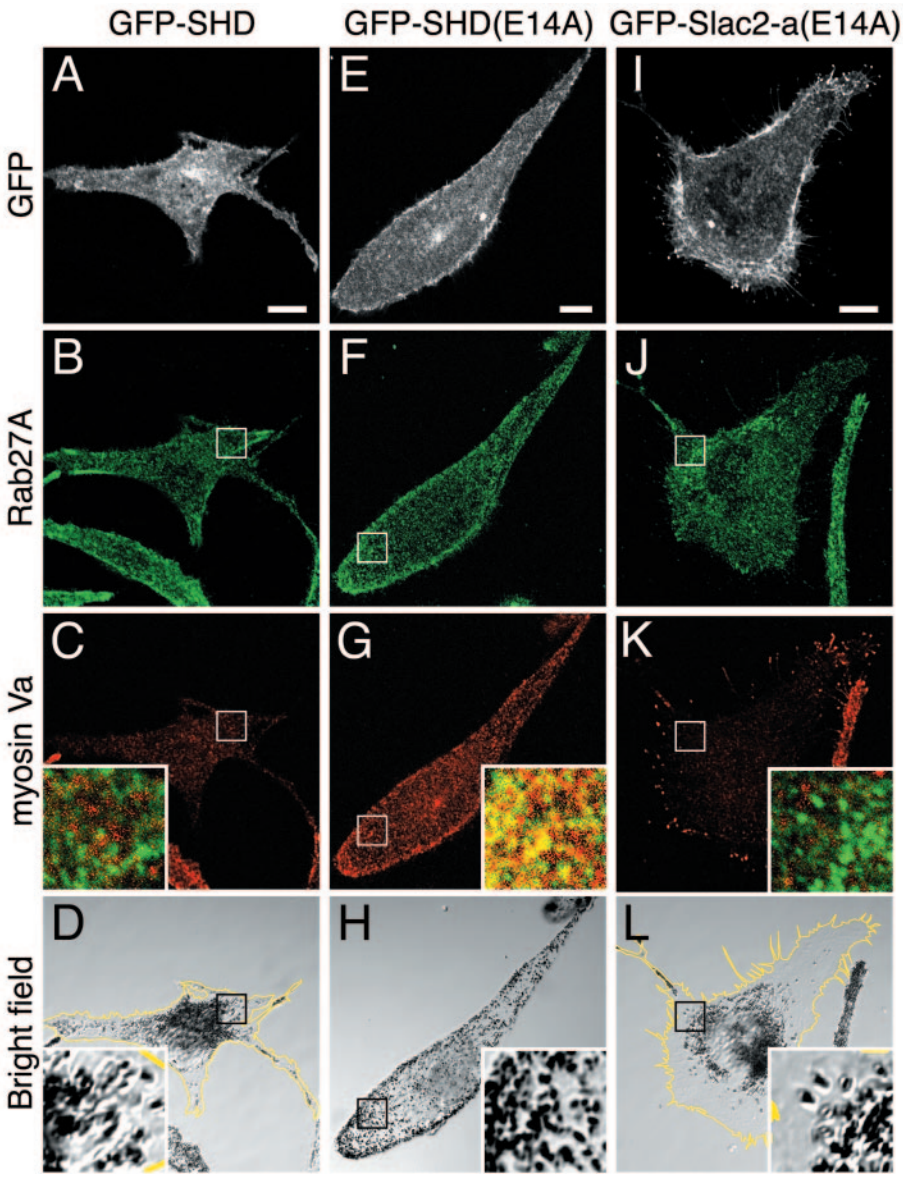

FIG. 4. Effect of Rab27A-binding-defective mutants of Slac2-a on melanosome transport. Melan-a cells were transfected with vectors encoding GFP-SHD (A to D), GFP-SHD(E14A) (E to H), or GFP–Slac2-a(E14A) (I to L). After cells were fixed and permeabilized, they were stained with anti-Rab27A and anti-myosin Va antibodies followed by Alexa Fluor secondary antibody conjugates. Fluorescence of GFP–Slac2-a mutants (A, E, and I), Rab27A (B, F, and J), and myosin Va (C, G, and K) was analyzed by confocal microscopy. Bright-field images (D, H, and L) show the melanosome distribution in the cells, and the cells are outlined in yellow (D and L). The insets in panels C, G, and K are merged images of Rab27A and myosin Va, and bright-field images corresponding to each inset are shown in panels D, H, and L, respectively. Note that expression of GFP-SHD induced melanosome aggregation in the perinucleus (D) and segregation of Rab27A from melanosomes and myosin Va (inset in panel C), whereas GFP-SHD(E14A) had no effect on either melanosome distribution (H) or colocalization of Rab27A with myosin Va on melanosomes (yellow signals in the inset in panel G and melanosome distribution in the inset in panel H). Expression of GFP–Slac2-a(E14A) also caused melanosome accumulation in the perinucleus (L) and reduced myosin Va signals (K). Bars, 10 μm.

that lacks the C-terminal half (481 to 590 amino acids) of the ABD of Slac2-a (named ΔABD). As shown in Fig. 2D (lane 4 in third panel), the ΔABD (still containing the SHD and MBD) bound normally to Rab27A and myosin Va, but binding to actin was completely abolished. If the function of Slac2-a is limited to the linkage between Rab27A and myosin Va, the ΔABD should support appropriate melanosome transport at the cell periphery because it forms a complex with Rab27A and myosin Va (Fig. 2D, lane 4 in first and second panels). When GFP-ΔABD was expressed in melan-a cells, the ΔABD induced clear accumulation of melanosomes in the perinuclear region (89.5% ± 2.7%) and decreased immunoreactivity of myosin Va (Fig. 6E to H and 7), demonstrating the physiological importance of the ABD of Slac2-a in melanosome transport. Since the full-length point mutants of Slac2-a [Slac2-a (E14A) and Slac2-a(EA)] strongly induced melanosome aggregation in melanocytes (Fig. 4I to L and 5I to L), we tested the effect of expression of the full-length actin-binding mutant

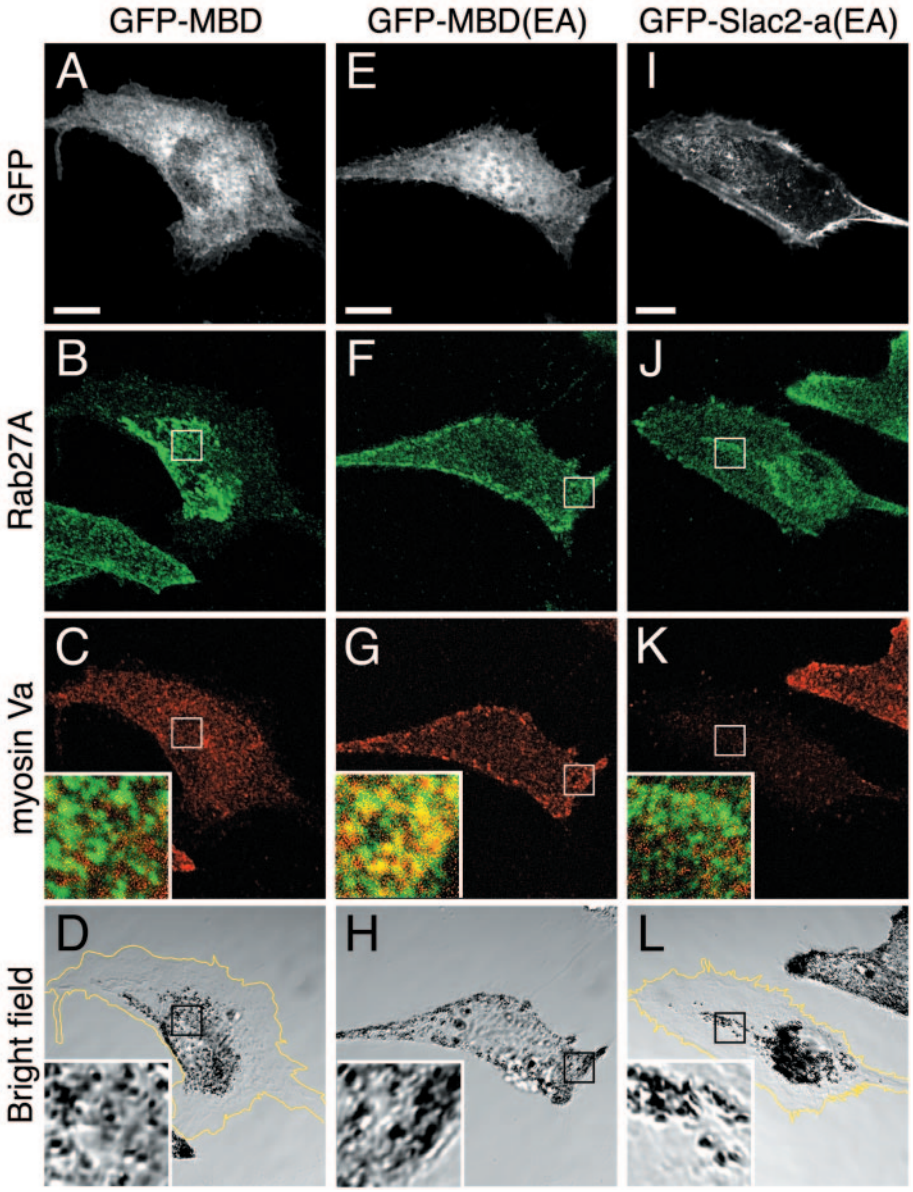

FIG. 5. Effect of myosin Va-binding-defective mutants of Slac2-a on melanosome transport. Melan-a cells were transfected with vectors encoding GFP-MBD (A to D), GFP-MBD(EA) (E to H), or GFP–Slac2-a(EA) (I to L). After cells were fixed and permeabilized, they were stained with anti-Rab27A and anti-myosin Va antibodies followed by Alexa Fluor secondary antibody conjugates. Fluorescence of GFP–Slac2-a mutants (A, E, and I), Rab27A (B, F, and J), and myosin Va (C, G, and K) was analyzed by confocal microscopy. Bright-field images (D, H, and L) show the melanosome distribution in cells, and the cells were outlined in yellow (D and L). The insets in panels C, G, and K are merged images of Rab27A and myosin Va, and bright-field images corresponding to each inset are shown in panels D, H, and L, respectively. Note that expression of GFP-MBD induced melanosome aggregation in the perinucleus (D) and segregation of myosin Va from Rab27A and melanosomes (inset in panel C), whereas GFP-MBD(EA) affected neither melanosome distribution (H) nor colocalization of Rab27A with myosin Va on melanosomes (yellow signals in the inset in panel G and melanosome distribution in the inset in panel H). Expression of GFP–Slac2-a(EA) also caused melanosome accumulation in the perinucleus (L) and reduced myosin Va signals (K). Bars, 10 μm.

Slac2-a(KA) on melanosome transport. As expected, high percentages (89.3% ± 4.4%) of melan-a cells expressing GFP–Slac2-a(KA) exhibited perinuclear accumulation of melanosomes (Fig. 6I to L and 7), and down-regulation of endogenous myosin Va expression levels seemed to occur. Taken together, these results indicate that formation of a tripartite protein complex between Rab27A, Slac2-a, and myosin Va is essential but not sufficient for melanosome transport and that the actin-binding ability of the ABD of Slac2-a is also necessary for normal melanosome distribution at the cell periphery.

DISCUSSION

We and others previously discovered that the SHD and MBD of Slac2-a function as a specific Rab27A-binding domain and a myosin Va-binding domain, respectively, and as a result

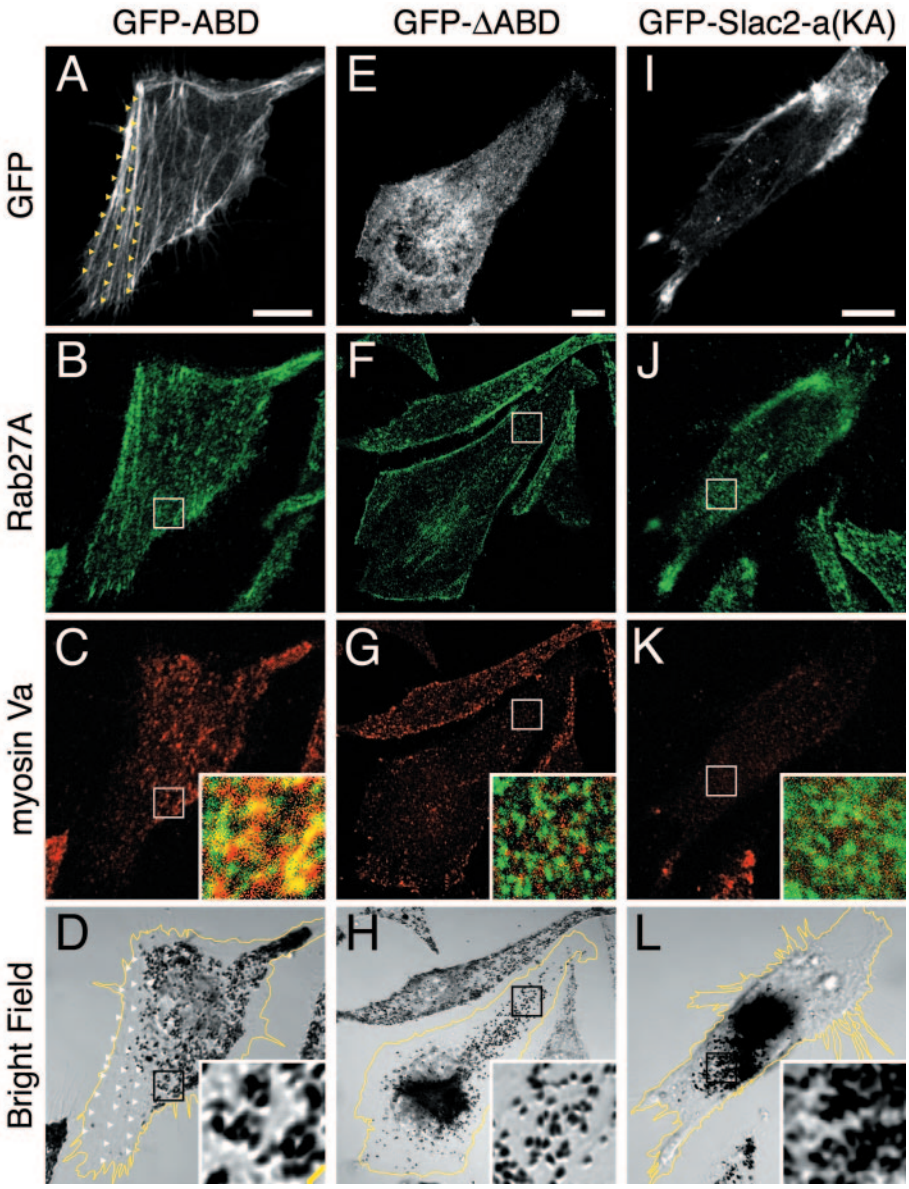

FIG. 6. Effect of actin-binding-defective mutants of Slac2-a (KA and ΔABD) on melanosome transport. Melan-a cells were transfected with vectors encoding GFP-ABD (A to D), GFP-ΔABD (E to H), or GFP–Slac2-a(KA) (I to L). After cells were fixed and permeabilized, they were stained with anti-Rab27A and anti-myosin Va antibodies followed by Alexa Fluor secondary antibody conjugates. Fluorescence of GFP–Slac2-a mutants (A, E, and I), Rab27A (B, F, and J), and myosin Va (C, G, and K) was analyzed by confocal microscopy. Bright-field images (D, H, and L) show the melanosome distribution in cells, and the cells were outlined in yellow (D, H, and L). The insets in panels C, G, and K are merged images of Rab27A and myosin Va, and bright-field images corresponding to each inset are shown in panels D, H, and L, respectively. Note that expression of GFP-ABD did not alter distributions of Rab27A and myosin Va on melanosomes (yellow signals in the insets in panel C and melanosome distribution in the inset in panel D) but did result in exclusion of melanosomes from peripheral actin bundles without large clumps of melanosomes around the nucleus (A and D, arrowheads). Expression of GFP-ΔABD and GFP–Slac2-a(KA) caused melanosome accumulation in the perinucleus (H and L) and reduced myosin Va signals (G and K) even though these mutants still retained the ability to bind to both Rab27A and myosin Va (Fig. 2D and data not shown). These findings indicate that formation of a tripartite protein complex consisting of Rab27A, Slac2-a, and myosin Va is not sufficient for melanosome transport and that the actin-binding ability of ABD is also necessary for normal melanosome distribution at the cell periphery. Bars, 10 μm.

Slac2-a is now widely believed to act as a linker protein between Rab27A and myosin Va in melanosome transport (i.e., by formation of a tripartite protein complex composed of Rab27A, Slac2-a, and myosin Va) (18, 34, 41, 46, 51). However, the precise molecular mechanism underlying the recognition of Rab27A or myosin Va by Slac2-a in vivo was poorly understood. In the present study, we demonstrated the physiological significance of three functional domains of Slac2-a, i.e., the SHD, MBD, and ABD, in actin-based melanosome transport in melanocytes by Ala-based site-directed mutagenesis (Fig. 1 and 2) in combination with cellular assay for melanosome distribution (Fig. 3 to 6). We identified for the first

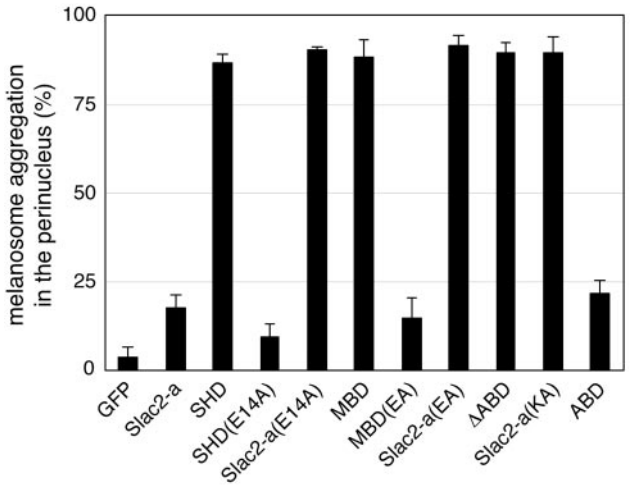

FIG. 7. Summary of melanosome distribution assay results. Melan-a cells were transfected with a vector encoding the indicated Slac2-a protein tagged with GFP. Images of transfected melan-a cells were captured at random by using GFP fluorescence as a marker, and we judged whether melanosomes had aggregated in the perinucleus by examining the corresponding bright-field images. The results are expressed as the percentages of cells exhibiting perinuclear melanosome aggregation and are means ± standard deviations from three independent experiments ($n > 150$).

time residues critical for binding to Rab27A (E-14 of Slac2-a) or myosin Va (D-378, E-380, E-381, and E-382 of Slac2-a) and succeeded in creating a Slac2-a point mutant that specifically impairs binding to either Rab27A or myosin Va. The expression of GFP-tagged Slac2-a point mutants in melan-a cells clearly showed that these mutant Slac2-a proteins (i.e., loss-of-function-type Slac2-a) behave as a dominant negative form in vivo (Fig. 7). The Slac2-a truncation mutants containing a single functional domain (SHD and MBD) and full-length point mutants unable to interact with one of the binding partners [Slac2-a(E14A) and Slac2-a(EA)] strongly induced melanosome accumulation in the perinucleus, whereas a single functional domain incapable of binding to its partner [SHD (E14A) and MBD(EA)] was neutral in terms of melanosome transport. We therefore concluded that the interaction between Rab27A and Slac2-a (or Slac2-a and myosin Va) is indeed essential for melanosome transport in vivo.

The most notable and surprising finding in this study is that formation of the tripartite protein complex (Rab27A–Slac2-a–myosin Va) alone is insufficient for appropriate melanosome transport and that involvement of the ABD of Slac2-a (most likely binding to actin) is also indispensable to this process. We previously found that the ABD of Slac2-a and Slac2-c directly interacts with actin in vitro and in cultured cells, and we hypothesized that actin binding to Slac2-a is one of the essential steps in melanosome transport (13). Consistent with this hypothesis, expression of Slac2-a mutants lacking actin-binding activity [ΔABD and Slac2-a(KA)] in melan-a cells greatly induced melanosome accumulation in the perinucleus (Fig. 6E to L and 7) even though these mutants still retained Rab27A- and myosin Va-binding activities (Fig. 2D and data not shown). Therefore, the dominant negative effect of the actin-binding-defective mutants of Slac2-a on melanosome transport is most likely attributable to specific loss of actin-binding activity rather than disruption of a Rab27A–Slac2-a–myosin Va complex, although the presence of an additional ABD ligand(s) cannot be completely ruled out. Since the basic cluster (K-493, R-495, R-496, and K-497) in the ABD of Slac2-a, which is critical for actin binding, is also conserved in Slac2-c, the closest isoform of Slac2-a, and since Slac2-c also possesses actin-binding activity (13), it is highly possible that Slac2-c does not just function as a linker protein between Rab27A and myosin Va/VIIa and that additional actin-binding activity in the ABD of Slac2-c is important to expressing its function in membrane transport, such as retinal melanosome transport (6). Actually, the ABD of Slac2-c has recently been shown to tether secretory granules to actin filaments to facilitate granule exocytosis independent of myosin Va/VIIa function in pancreatic β-cells (L. Waselle, T. Coppola, M. Fukuda, M. Iezzi, A. El-Amraoui, C. Petit, and R. Regazzi, submitted for publication).

How does the Slac2-a–actin complex control actin-based melanosome transport? We think that Slac2-a binding to actin may be involved in three steps of melanosome transport. The first step is the melanosome transfer from microtubules to actin filaments after microtubule-dependent melanosome transport. Since this step is accomplished by the Rab27A–Slac2-a–myosin Va complex, one possible function of the Slac2-a–actin interaction is to facilitate trapping of a preformed tripartite protein complex on actin filaments or formation of a tripartite protein complex on actin filaments (i.e., the Rab27A–Slac2-a complex on melanosomes is trapped on actin filaments and myosin Va is then recruited to initiate actin-based melanosome transport) (Fig. 8A). The second step is the melanosome transport along actin filaments. Another possible function of Slac2-a–actin interaction is as an accelerator of transport or a stabilizer of a transport complex during actin-based transport driven by the myosin Va-actin machinery (Fig. 8B). The third step is the melanosome capture by peripheral actin filaments. At the end of the actin-based transport, melanosomes must be retained on peripheral F-actin before transfer to adjacent keratinocytes, and thus it is also possible that Slac2-a binds peripheral F-actin and makes melanosomes captured by actin filaments near the plasma membrane (Fig. 8C).

Involvement of Slac2-a in the melanosome transfer step (Fig. 8A) is easily monitored with high sensitivity under our experimental conditions, because actin-based transport is a unidirectional movement, and once the melanosome transfer step is inhibited, melanosomes return to the cell center by bidirectional microtubule transport (i.e., accumulation of melanosomes in the perinucleus). Since expression of actin-binding-deficient mutants of Slac2-a strongly inhibited melanosome transition from microtubules to actin filaments (i.e., melanosomes accumulated in the perinucleus) (Fig. 6E to L), the actin-binding ability of Slac2-a must be required for the melanosome transfer step. By contrast, the actin-based transport process itself (Fig. 8B) and melanosome capture by actin filaments (Fig. 8C) are difficult to observe because of being a short-range movement, and it is impossible to exclude the possibility that Slac2-a also participates in the actin-based transport or the capture mechanism. Further work is necessary to elucidate this issue.

Although our data presented in this paper clearly point to the functional involvement of three domains of Slac2-a (the SHD, MBD, and ABD) in actin-based melanosome transport,

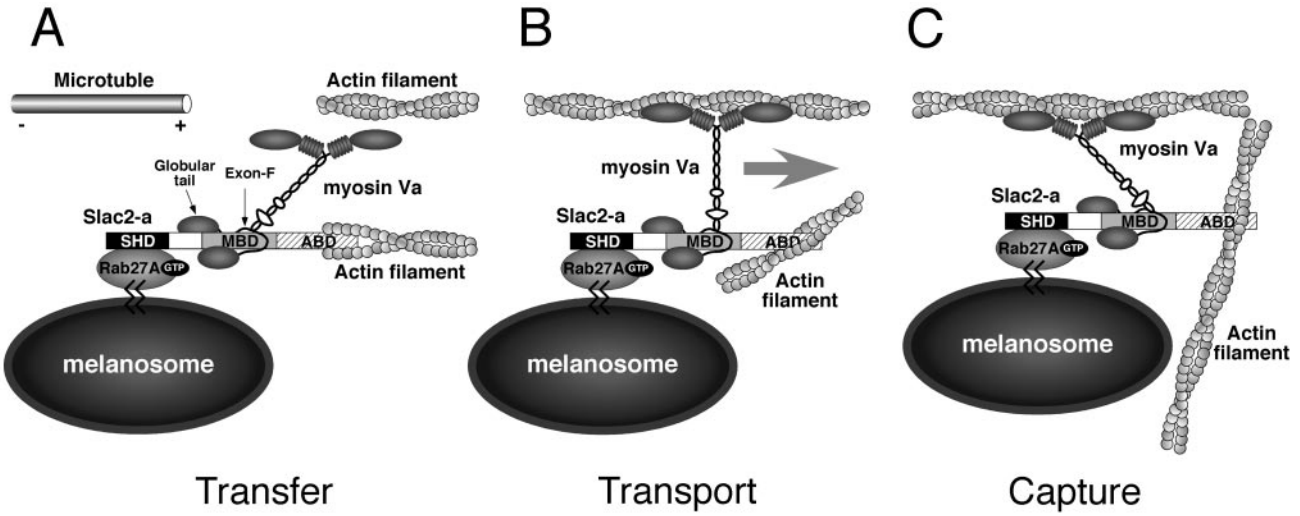

FIG. 8. Schematic model illustrating possible roles of Slac2-a–actin interaction in actin-based melanosome transport. Slac2-a binding to actin could be involved in the following three steps of melanosome transport: melanosome transfer from microtubules to actin filaments (A), melanosome transport along actin filaments (B), and melanosome capture by actin filaments near the plasma membrane of the cell (C). Note that these three steps are not mutually exclusive. In this study we demonstrated that association between Slac2-a and actin is required for the transfer step (A). See Discussion for details.

especially at the melanosome transfer step, the functional relationships between the domains (especially between the tripartite protein complex and the Slac2-a–actin complex) have not been thoroughly resolved. For instance, the ABD (truncated form of Slac2-a, amino acids 401 to 590 of Slac2-a) tightly binds actin, whereas the full-length Slac2-a has relatively weak affinity for actin (13), suggesting that Slac2-a–actin interaction may be affected (or regulated) by binding of the SHD to Rab27A and/or binding of the MBD to myosin Va. Further three-dimensional analysis of the tripartite protein complex is required to address this issue.

In summary, we analyzed three functional domains of Slac2-a, the SHD, MBD, and ABD, in vivo and demonstrated that formation of a tripartite protein complex between Rab27A, Slac2-a, and myosin Va is essential but not sufficient for melanosome transfer from microtubules to actin filaments and that the function of the ABD (i.e., actin binding) of Slac2-a is indispensable for this process. The findings of this study will greatly promote our understanding of the organelle transport system driven by concerted action between the Rab protein, Rab effector, and motor protein. Our study also provides useful tools for studying the function of Slac2-a (or Slac2-c) in other cell types.

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